Neuroprotective–Neurotrophic Effect of Endogenous Dehydroepiandrosterone Sulfate During Intense Stress Exposure

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ABSTRACT

Recent reports demonstrate neurotrophic properties of dehydroepiandrosterone sulfate (DHEAS) in men at rest, as well as profound neurotrophic responses to stress in both men and women. Little is known of neuroprotective–neurotrophic effects of DHEAS during stress exposure, either in men or women. This translational study was designed to examine neuroprotective–neurotrophic effects of DHEAS throughout intense stress exposure in healthy men and women. The study took place within a stressful 12-day military survival course. Utilizing a longitudinal cross-sectional repeated measures design, One hundred sixteen healthy active-duty military personnel (80% male) were studied before, during, and 24 h after the course. The dependent variable was the neurotrophin salivary nerve growth factor (sNGF). In terms of total hormone output, the effect of DHEAS on sNGF was mediated by testosterone. Unlike testosterone or cortisol, DHEAS reliably predicted sNGF at each time point, and change in DHEAS predicted change in sNGF across time points. Baseline DHEAS predicted total sNGF output across the stress trajectory. Consistent with preclinical as well as cross-sectional human research, this study demonstrates neuroprotective–neurotrophic effects of DHEAS in healthy men and women exposed to intense stress. Results are evaluated in relation to established criteria for causation.

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1. Introduction

Neurotrophins are proteins found within a broad range of cell types in the brain and periphery that facilitate neuronal growth, survival, and plasticity [1]. The neurotrophin "superfamily" includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), neurotrophin-4/5 (NT4/5), and neurotrophin-6 [2,3]. Target tissues are hypothesized to regulate neuron survival by making neurotrophins available in limited

amounts, resulting in selection of neurons with the best connectivity to the target tissue. NGF, in particular, is released by the target tissue and taken up in responsive neurons by receptor-mediated endocytosis. It is then transported retrogradely into the cell where it exerts trophic effects [2,4]. Lu et al. [3] proposed a "Yin and Yang model," whereby neurotrophic action is mediated by two principal classes of transmembrane receptor systems: the tyrosine kinase (Trk) receptors (including TrkA [selective for NGF], TrkB [selective for BDNF and NT4/5], and TrkC [selective for NT3]) and the neurotrophin receptor p75^{NTR}. Each receptor type binds mature neurotrophins and/or neurotrophin precursors (proneurotrophins), creating a complex "balance" that then causes neuronal survival or death [5,6].

Accruing evidence shows that neurotrophins are responsive to stress exposure in both animals [7] and humans [8–11]. Early work [7], for example, showed that intermale fighting behavior in mice elicited entry of NGF from the submandibular gland into the bloodstream, reaching peak levels within 3 h. In human research, Aloe and colleagues [8] employed a novice parachute jumping model, and found that blood NGF levels increased 84% the night prior to
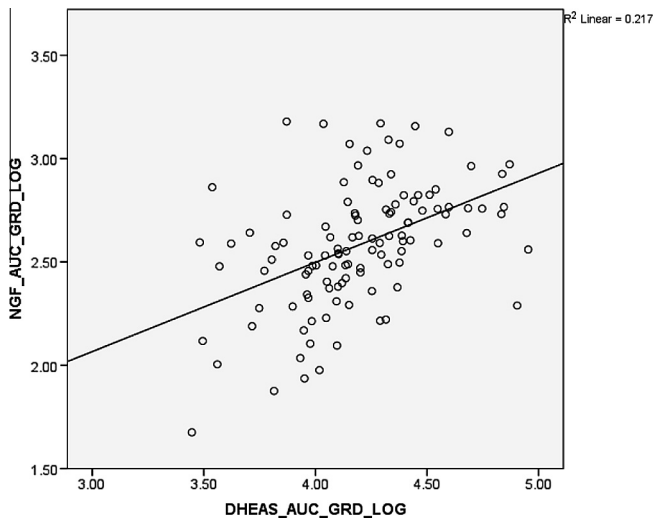
Fig. 1. Positive association of DHEAS total output and sNGF total output.
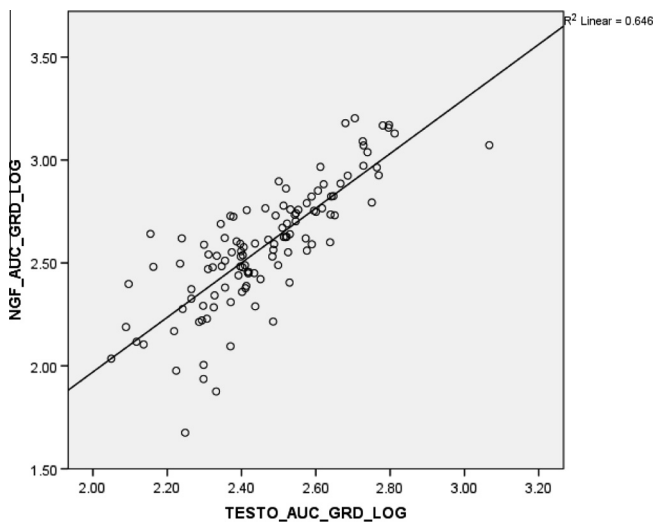

Fig. 2. Positive association of testosterone total output and sNGF total output.

a jump and 107% postjump. More recent studies of sNGF report substantial reactivity and residual elevation in response to interpersonal conflict stress [9,10], as well as mock-captivity stress during military survival training [11].

There is also evidence that steroid hormones modulate neuroprotective–neurotrophic activity, attributable in part to direct interfaceY with neurotrophins [12,13]. Estradiol, for example, modulates protein phosphatases, phosphorylation of signaling kinases, and mitochondrial regulation [14], while both estradiol [15] and progesterone [16] are believed to interact with (or express molecules that interact with) neurotrophins to promote neuronal survival. Testosterone, in turn, may exert neurotrophic effects via aromatization to estradiol and/or by transformation into 5α-dihydrotestosterone [17]. Also, the testosterone precursor dehydroepiandrosterone (DHEA) and its sulfate ester DHEA sulfate (DHEAS) appear to promote neuroprotection [18], neurite growth [19], and neurogenesis [20]. Regarding neuroprotection, DHEA(S) is thought to exert pro-survival effects by modulating gamma-aminobutyric acid [18], glutamate [21], N-methyl-ᴅ-aspartate, sigma-1 receptors [22], or after conversion to testosterone [5]. DHEA also operates in conjunction with NGF via at least two different mechanisms. In the first, DHEA binds with TrkA and p75^{NTR} on sensory

and/or sympathetic neurons, the balance of which ignites events modulating apoptosis [5]. In the second, DHEA has been shown to evoke NGF mRNA expression in target cells [23]. In a study of pregnant women, Schulte-Herbrüggen et al. [24] showed no relationships between serum DHEAS and NGF. In contrast, we showed that DHEAS independently associated with salivary NGF (sNGF) in military men under baseline conditions [25], while DHEA did not.

We now know that both DHEA(S) [26] and NGF [7–11] respond affirmatively to stressful insult, yet the association between these analytes during stress exposure is not understood. Characterization of this relationship has implications for prevention and treatment of traumatic stress and injury [13], degenerative disease management [12], and nerve repair [27]. In this report, we extended our prior study of neuroprotective properties of DHEAS in men under baseline conditions [25] to a prospective paradigm involving intense stress exposure in both men and women. We hypothesized that (a) robust associations would prevail between total output of DHEAS and sNGF across the stress trajectory and at each time point, (b) *changes* in DHEAS would predict corresponding changes in sNGF, and (c) *baseline* DHEAS would positively predict total sNGF output across the stress trajectory. We also explored the roles of testosterone and cortisol. In light of less definitive prior literature, directional hypotheses were not stated regarding these analytes.

2. Experimental

Survival, Evasion, Resistance, and Escape (SERE) training is described in earlier reports, [11,26,28,29], while the participants and methodology for the current study are detailed in Taylor et al. [11]. Growing evidence confirms that SERE is an intensely stressful event, quantified by severe disruption of physiological [28] and self-report indices [29]. This protocol was approved by the Naval Health Research Center Institutional Review Board, and all subjects signed written informed consent.

2.1. Protocol

Participants completed baseline salivary assessments on the first day of the academic phase of SERE training (Time 1 [T1]; baseline). Subsequently, all subjects experienced a rigorous evasion exercise, and then participated in a highly realistic mock-captivity scenario. Assessments were performed again directly after a stressful mock-captivity event (Time 2 [T2]; mock-captivity stress). Finally, approximately 24 h after release from mock captivity (marking completion of field training), assessments were completed a third time (Time 3 [T3]; recovery). The entire course lasted 12 days.

2.2. Salivary assessments

A salivary sample was obtained via the passive drool method [30] between 1145 h and 1247 h under baseline, free-living conditions on the first day of academic (classroom) instruction for military survival training. After data collection, all samples were immediately placed on dry ice and transferred to Salimetrics, LLC (State College, PA) for storage and data processing. Samples were assayed for NGF, DHEAS, testosterone, and cortisol. The NGF assay was performed in triplicate using a commercially available enzyme immunoassay kit (Promega NGF Emax ImmunoAssay Systems, Madison, WI) modified for use with saliva. The standard curve measured NGF from 3.9 to 250 pg/mL. The assay has an intra-assay precision of 14.5% and an inter-assay precision of 15.5%. Recovery of NGF added to saliva samples averaged 95.3%. Linearity ranged from 82.3% to 127.2%. Mean ± SE baseline sNGF concentrations in this sample were 159.9 ± 12.8 pg/mL. Samples were assayed for

salivary DHEAS in duplicate using a highly sensitive enzyme immunoassay. The test uses 100 μL of saliva per determination, has a lower limit of sensitivity of 43 pg/mL, standard curve range from 189 pg/mL to 15,300 pg/mL, an average intra-assay coefficient of variation of 7.3%, and an inter-assay coefficient of variation of 7.6%. Method accuracy determined by spike recovery averaged 105.9%, and linearity determined by serial dilution averaged 98.2%. Mean ± SE baseline DHEAS concentrations were 4370.8 ± 330.5 pg/mL. The testosterone assay was also performed in duplicate using a highly sensitive enzyme immunoassay. The test uses 25 μL of saliva per determination, has a lower limit of sensitivity of 1.0 pg/mL, standard curve range from 6.1 pg/mL to 600 pg/mL, an average intra-assay coefficient of variation of 4.6%, and an average inter-assay coefficient of variation of 9.8%. Method accuracy determined by spike recovery averaged 104.3% and linearity determined by serial dilution averaged 102.4%. Serum–saliva correlations from a normative database (Salimetrics) of male subjects is high ($r = .91$, $p < .001$, $n = 26$). Mean ± SE baseline testosterone concentrations in this sample were 148.7 ± 8.6 pg/mL. Salivary cortisol was assayed in duplicate using a highly sensitive enzyme immunoassay (Salimetrics). The test uses 25 ml of saliva per determination, has a lower limit of sensitivity of 0.003 mg/dl, standard curve range from 0.012 mg/dL to 3.0 mg/dL, an average intra-assay coefficient of variation of 3.5%, and an average inter-assay coefficient of variation of 5.1%. Method accuracy determined by spike recovery averaged 100.8%, and linearity determined by serial dilution averaged 91.7%. Mean ± SE baseline cortisol concentrations in this sample were 0.2 ± 0.01 μg/dL.

2.3. Statistical analysis

Data were analyzed using SPSS software version 19.0 (IBM, Armonk, New York). Distribution characteristics for all continuous variables were examined to determine if assumptions of normality were met, following conservative predefined limits (e.g., skewness between −1 and 1 [31], kurtosis between −3 and 3). Variables exceeding any of these limits were log-transformed prior to performing the relevant statistical test. All data transformations reduced skewness and kurtosis to acceptable levels. Untransformed means are reported for ease of interpretation. Absolute (value 2 − value 1) and relative Δ scores ([value 2 − value 1/value 1] × 100%) were also computed and used to operationally define "reactivity" (i.e., initial response from baseline to mock-captivity stress), "recovery" (i.e., change from mock-captivity stress to 24-h recovery), and "residual elevation," (i.e., sustained disruption from baseline to 24-h recovery) [11,28]. Descriptive analyses were conducted to summarize subject characteristics. For each hypothesis test, a theoretically relevant variable (e.g., age, sex, education, or body mass index [BMI]) was selected as a covariate if it was significantly related to at least one independent variable and the dependent variables of interest (both $p < 0.05$), thus qualifying as a potential confounder [32]. Separate linear regression models (including covariates, where applicable) evaluated the unique and combined influence of DHEAS, testosterone, and cortisol on sNGF with respect to total hormone output (measured by area under the curve with respect to ground [AUC_G]), each time point (baseline, stress, and recovery), and each change index (reactivity, recovery, and residual elevation/depression). Finally, temporality was explored by evaluating effects of baseline DHEAS, testosterone, and cortisol on total sNGF output across the stress trajectory. Collinearity diagnostics were performed to confirm assumptions were met for each regression model, using a conservative variable inflation factor cut point of 3.0 [33]. All formal hypothesis tests were two-sided, and the probability of committing a Type I error was set at 0.05. It was acknowledged when more stringent alpha levels were achieved ($p < 0.01$ or $p < 0.001$).

3. Results

Overall effects of survival stress exposure on sNGF and cortisol in this sample are detailed in Taylor et al. [11], while the effects of survival stress exposure on DHEAS and testosterone are also detailed in a separate report (Rauh et al., manuscript in preparation).

3.1. Neuroprotective-neurotrophic effects of DHEAS

In the first regression model, total hormone output (AUC_G) of the independent variables (DHEAS, testosterone, and cortisol) combined to explain 63.7% of variance in sNGF output ($F = 65.4$, $p < 0.001$). Standardized beta coefficients revealed that testosterone exerted an independent effect ($β = 0.80$, $p < 0.001$), while the other predictors were not significant. In light of this unexpected finding, we then used regression-based causal steps modeling [34] to evaluate whether testosterone mediated a hypothesized direct effect of DHEAS on sNGF. Following this approach, DHEAS predicted sNGF in an initial regression model ($β = 0.45$, $p < 0.001$). When testosterone was added, the direct effect of DHEAS (path c′) on sNGF was nearly eradicated and no longer significant ($β = .04$, $p = .57$), thus suggesting a mediated effect. An alternate statistical test (Sobel Test; 34) evaluating the hypothesized difference between the total effect (path c) and the direct effect (path c′) of DHEAS on sNGF produced a similar result (test statistic = 4.0, $p < 0.001$). Fig. 1 depicts positive association of DHEAS to sNGF, while Fig. 2 depicts Positive association of testosterone to sNGF.

The models were then decomposed at each time point. At baseline, the independent variables (DHEAS, testosterone, and cortisol) combined to account for 10.2% of variance in sNGF ($F = 5.3$, $p < 0.01$). Standardized beta coefficients showed that DHEAS exerted an independent effect on sNGF ($β = 0.39$, $p < 0.001$), while the other predictors were not significant. During stress exposure, the independent variables combined to account for 28.0% of variance in NGF ($F = 15.8$, $p < 0.001$). Again, DHEAS exerted an independent effect ($β = 0.56$, $p < 0.001$) while the other predictors were not significant. During recovery, the predictor set accounted for 18.0% of variance in sNGF ($F = 9.2$, $p < 0.001$), and DHEAS exerted an independent effect ($β = 0.47$, $p < 0.001$) while the other predictors did not.

The models were then decomposed relative to each change index. In terms of reactivity, the independent variables (DHEAS, testosterone, and cortisol reactivity) and covariate (sex) combined to account for 20.3% of variance in sNGF reactivity ($F = 8.2$, $p < 0.001$). Standardized beta coefficients revealed that DHEAS reactivity exerted an independent effect ($β = 0.39$, $p < 0.001$), while the other predictors were not significant. In terms of recovery, the predictors combined to account for 28.2% of variance in sNGF recovery ($F = 15.5$, $p < 0.001$); DHEAS recovery exerted an independent effect ($β = 0.52$, $p < 0.001$), as did testosterone recovery ($β = −0.27$, $p < 0.01$). In terms of residual elevation/depression, the independent variables explained 12.4% of variance in sNGF residual elevation ($F = 6.2$, $p < 0.001$). DHEAS residual elevation exerted an independent effect ($β = 0.35$, $p < 0.001$), while the other predictors did not.

3.2. Temporal relationships

To characterize temporal relationships, we examined the relative contributions of *baseline* DHEAS, testosterone and cortisol to total sNGF output across the stress trajectory. The independent variables combined to account for 10.5% of variance in sNGF output ($F = 5.5$, $p < 0.01$). Standardized beta coefficients showed that baseline DHEAS exerted an independent effect on total sNGF output ($β = 0.36$, $p < 0.001$), while the other predictors were not signifi-

cant. Testing the alternate directional hypothesis (that baseline sNGF predicts total DHEAS output), a separate regression model was performed with baseline sNGF as the independent variable and total DHEAS output as the dependent variable. This model accounted for 5.1% of variance in DHEAS output ($F = 7.0$, $p < 0.01$), and the standardized beta coefficient ($\beta = 0.24$, $p < 0.01$) was attenuated by 33.3%.

4. Discussion

The purpose of this study was to evaluate neuroprotective-neurotrophic effects of DHEAS throughout intense stress exposure in healthy men and women. In terms of total hormone output, the unique effect of DHEAS on sNGF was mediated by testosterone. Unlike testosterone or cortisol, DHEAS reliably predicted sNGF at each time point; furthermore, change in DHEAS predicted change in sNGF across time points. DHEAS also provided the best evidence for temporality, with baseline DHEAS predicting total sNGF output across the stress trajectory. These findings are discussed within the framework of Hill's [35] criteria for causality.

4.1. Strength of association

A larger association is more supportive of a case for causality. Although the observed associations between DHEAS and sNGF in this study were robust, they were still of moderate magnitude, suggesting that causality should be considered cautiously. The association between testosterone total output and sNGF total output was the strongest in this study, for which biologically plausible mediated effects were identified.

4.2. Consistency

Consistent findings observed by different scientists in different places with different samples strengthens the likelihood of causality. In addition to the preclinical literature linking DHEA(S) to NGF [23], the current findings are comparable to our prior report. Specifically, the baseline association of DHEAS to sNGF in this study (adjusted for testosterone and cortisol) replicates the independent association of DHEAS to sNGF (adjusted for DHEA) in a separate, demographically similar sample of healthy military men [25]. Replication of the current study under stressful conditions is needed to further evaluate the consistency of these findings.

4.3. Specificity

If sNGF endpoints varied specifically as a function of DHEAS (and not other candidate predictors), that could be suggestive of causality. In this study, only two other candidate predictors were evaluated (testosterone and cortisol), and testosterone convincingly associated with sNGF in terms of total output. However, evidence was provided that testosterone mediated the direct effect of DHEAS on sNGF. As alluded to above, this is a biologically plausible explanation in light of the well-characterized steroidogenic pathway [36]. Future work is needed to further explore other variables that could explain variance in sNGF.

4.4. Temporality

The temporal nature of an association is an important clue to causality. In this longitudinal repeated measures study, we showed that baseline DHEAS predicted total sNGF output more convincingly than baseline sNGF predicted total DHEAS output. However, both analyses were significant; therefore, neither can be dismissed, and bidirectional associations are certainly possible.

4.5. Biological gradient

If causality exists, greater exposure should confer a greater effect. In this study, strength of association between DHEAS and sNGF increased from baseline to stress exposure by 63.6%, coinciding with substantial reactivity of both analytes (sNGF: +136.9%, DHEAS: +268.5%). Likewise, strength of association decreased upon removal of stress, yet remained stronger than that observed at baseline. This corresponds to the residual elevation observed in both analytes whereby recovery values exceeded that of baseline (sNGF: +67.4%, DHEAS: +143.0%). We were unable to evaluate varying *doses* (i.e., intensities) of stress exposure, which would have offered more discriminating evidence of a biological gradient. That said, the consistent links shown between reactivity, recovery, and residual elevation of DHEAS and sNGF support the hypothesis that sNGF is sensitive to, and therefore a function of, changes in DHEAS.

4.6. Plausibility

A plausible biological mechanism is essential to the case for causality. Although DHEA(S) has been shown to modulate NGF receptor activity in sensory and sympathetic neurons [5], their *shared action* still does not fully explain the consistent positive associations in DHEAS and sNGF *concentrations* observed in this study. That DHEA(S) induces NGF expression [23,27], however, does imply that increases in DHEA(S) lead to proportional increased production of sNGF. The precise molecular mechanisms explaining the DHEA(S)-NGF interface, however, are still under investigation [37,38] which precludes definitive conclusions.

4.7. Coherence

Causality is clearest in the absence of competing theories or rival hypotheses [35]. Future studies at the basic and translational level are needed to dispassionately test alternate explanations of the observed link between DHEAS and sNGF. In other words, a causal relationship between DHEAS and sNGF is not the only available explanation for the observed associations.

4.8. Experiment

Experimental evidence does suggest that DHEA(S) treatment yields neurogenesis [20] and neuroprotection [18], as well as NGF overproduction in brain cortex [27]. Likewise, as discussed above, DHEA induces NGF expression in astrocyte/neuronal cell cultures [23]. Our translational findings, then, are consistent with the available experimental literature suggesting that DHEA(S) evokes NGF production.

4.9. Analogy

Similar observations under different scientific paradigms strengthen the case for causality. The current findings are consistent with the available preclinical and in vitro literature linking DHEA(S) to NGF, suggestive of a recurring theme across distinct scientific paradigms.

There are some limitations of this study. Importantly, causality cannot be implied from any single criterion alone, nor can it be definitively determined from any single study. Rather, use of Hill's criteria simply allows one to "build a case" for causality via accrued, convergent evidence. Also, as acknowledged in Taylor et al. [11] and Laurent et al. [10], some sNGF sample absorbance values fell outside the curve linearity. This leaves the possibility that some non-NGF material may have been present in saliva after stress, which could increase the unspecific binding and, as a result,

overestimate stress-induced sNGF concentrations. The sNGF assay is a new development, and it is currently undergoing refinement to enhance its precision.

In sum, this translational study reliably linked DHEAS to sNGF throughout stress exposure in healthy men and women. Evaluating the findings within Hill's criteria for causality, support was indicated for causal relationships across many but certainly not all criteria—thus conjuring the idiom, "the "jury is still out.""

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14. ABSTRACT

Context: Recent reports demonstrate neurotrophic properties of dehydroepiandrosterone sulfate (DHEAS) in men at rest, as well as profound neurotrophic responses to stress in both men and women. Little is known of neuroprotective-neurotrophic effects of DHEAS during stress exposure, either in men or women.
Objective: This translational study was designed to examine neuroprotective-neurotrophic effects of DHEAS throughout intense stress exposure in healthy men and women.
Setting: The study took place within a stressful 12-day military survival course.
Design: Participants were studied before, during, and 24 hours after the course.
Participants: One hundred sixteen healthy active-duty military personnel (80% male) were studied.
Main Outcome Measures: The dependent variable was the neurotrophin salivary nerve growth factor (sNGF).
Results: In terms of total hormone output, the effect of DHEAS on sNGF was mediated by testosterone. Unlike testosterone or cortisol, DHEAS reliably predicted sNGF at each time point, and change in DHEAS predicted change in sNGF across time points. Baseline DHEAS predicted total sNGF output across the stress trajectory.
Conclusion: Consistent with preclinical as well as cross-sectional human research, this study demonstrates neuroprotective-neurotrophic effects of DHEAS in healthy men and women exposed to intense stress. Results are evaluated in relation to Hill's criteria for causation.

15. SUBJECT TERMS
stress, military, survival, DHEA sulfate, nerve growth factor, allostasis

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